

# Egg Yolk Layers Vary in the Concentration of Steroid Hormones in Two Avian Species

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Maternally derived steroid hormones are known to be present in the yolks of avian eggs; however, the physiological mechanisms involved in their deposition remain largely unexplored. Investigations of steroid production by avian follicles have demonstrated temporal differences in the concentrations of progesterone, 17 $\beta$ -estradiol, and testosterone during yolk formation. Because yolk is deposited peripherally in concentric spheres as the oocyte develops, differences in the production of follicular hormones during yolk formation should be manifested in differences in the localization of steroids within layers of the yolk. To investigate this hypothesis we analyzed steroid hormone concentrations in layers of individual eggs of the dark-eyed junco (*Junco hyemalis*) and the red-winged blackbird (*Agelaius phoeniceus*). We found that in the dark-eyed junco the concentration of progesterone is significantly greater at the periphery of the yolk, while the concentration of 17 $\beta$ -estradiol is significantly greater near the center of the yolk. We also found in both the dark-eyed junco and the red-winged blackbird that the concentration of testosterone remains constant from the interior to the intermediate layers of the yolk and then drops sharply between the intermediate and exterior layers. The patterns of hormone localization that we found agree with those predicted by studies of temporal changes in steroidogenesis in the maturing follicle of the chicken, thus suggesting that within-yolk variation in yolk steroid concentrations in the dark-eyed

junco and the red-winged blackbird reflects temporal differences in the pattern of follicular steroidogenesis. Variation in the concentration of hormones among yolk layers presents a methodological concern for studies that involve the removal of yolk samples from viable eggs for subsequent hormonal analysis. This variation also has implications for the timing of embryonic exposure to steroid hormones. © 1999 Academic Press

**Key Words:** egg; yolk; follicle; progesterone; testosterone; estradiol; dark-eyed junco; red-winged blackbird.

Recent investigations have revealed the presence of maternally derived steroid hormones in the yolks of avian eggs (Schwabl, 1993; Adkins-Regan *et al.*, 1995; Schwabl *et al.*, 1997; Lipar *et al.*, 1999). The physiological mechanisms involved in the deposition of yolk steroids have not yet been fully elucidated. However, experimental elevation of steroid concentrations in maternal plasma results in a corresponding increase in steroid concentrations in the yolk (Adkins-Regan *et al.*, 1995; Schwabl, 1996a), indicating that the hormonal profiles of avian yolks reflect the concurrent hormonal status of the laying female.

Although the oocytes in a female bird contain some amount of yolk throughout most of her lifetime, most yolk deposition occurs rapidly over a period of 3 to 10 days (depending on the species) just prior to ovulation (King, 1973). The concentrations of steroid hormones in both the plasma and the follicles of females are known to undergo changes during this period of rapid yolk formation (Shahabi *et al.*, 1975; Wingfield and

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Farner, 1978; Donham, 1979; Hammond *et al.*, 1980; Johnsen and van Tienhoven, 1980; Sharp, 1980; Etches and Cheng, 1981; Bahr *et al.*, 1983). Yolk is deposited in concentric spheres around the periphery of the oocyte (Romanoff, 1960). Therefore, if steroid concentrations in the yolk reflect either follicular or plasma steroid concentrations in the female during yolk deposition, then one might expect to find variation in the concentration of steroid hormones among layers of yolk within an egg.

Bahr *et al.* (1983) found that in the domestic hen (*Gallus gallus*) the follicular concentrations of progesterone, testosterone, and  $17\beta$ -estradiol change as the follicle matures during the 5 days prior to ovulation. Moreover, the patterns of temporal change in the concentrations of each of these hormones differ markedly from one another (Fig. 1). Progesterone concentrations increase significantly during follicle development, while  $17\beta$ -estradiol concentrations decrease. Testosterone production increases slightly early in the period and then remains relatively constant before dropping off sharply during the last 24 h of yolk deposition. In addition, the absolute concentrations of each of the three hormones differ from one another, with progesterone present in greater amounts than either testosterone or  $17\beta$ -estradiol.

To determine whether there is variation in steroid

concentration among yolk layers of wild birds and, if so, whether it corresponds to the known pattern of follicular steroid production in chickens, we measured progesterone,  $17\beta$ -estradiol, and testosterone concentrations in eggs of the dark-eyed junco (*Junco hyemalis*). We also measured testosterone concentration in eggs of the red-winged blackbird (*Agelaius phoeniceus*). Based on the results of Bahr *et al.* (1983), if the reproductive physiology of these two species resembles that of the chicken, then we predicted that (a) progesterone levels would increase from the interior to the exterior layers of the yolk, (b)  $17\beta$ -estradiol levels would decrease from the interior to the exterior layers of the yolk, and (c) testosterone levels would increase from the interior to the intermediate layers and then decline in the outermost layers of the yolk. We also predicted that the absolute concentrations of each of these hormones would differ from one another as they do in the follicle of the domestic hen (Bahr *et al.*, 1983). Such results would support the hypothesis that yolk steroid concentrations mirror follicular steroid concentrations in the laying female. In addition, variation in steroid concentration among yolk layers, regardless of whether it reflects temporal variation in the follicular production of steroids, could lead to variation in the amount of hormone that the embryo is exposed to at different points during its development. Further, the presence of variation in hormone concentration within the yolk could mean that there are potential methodological problems in the protocol that is currently being used for the removal of yolk samples from viable eggs for subsequent hormonal analysis.

## METHODS

### General Methods

We collected 7 dark-eyed junco eggs from 7 nests located at or near the Mountain Lake Biological Station of the University of Virginia (approximately  $37^{\circ}14'N$ ,  $80^{\circ}25'W$ ) between 14 May and 2 June 1997. We also collected 17 red-winged blackbird eggs from 17 nests located near the Cedar Point Biological Station of the University of Nebraska (approximately  $41^{\circ}20'N$ ,  $101^{\circ}43'W$ ) between 18 May and 4 July 1997. We found nests while they were being built, visited them daily,

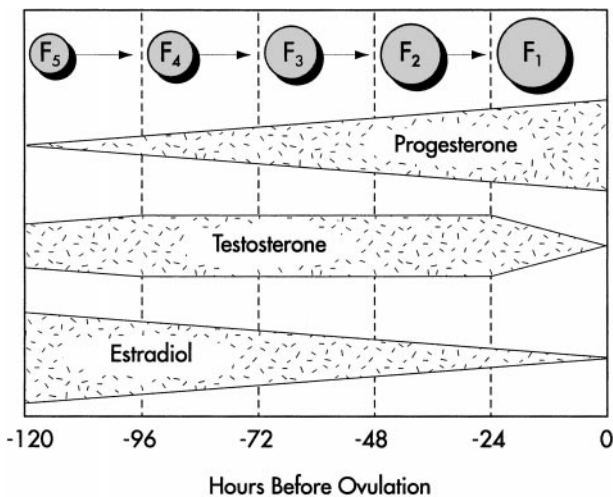


FIG. 1. A schematic representation of changes in follicular steroid concentrations during follicular maturation. F1 represents a follicle 1 day prior to ovulation, F2 represents a follicle 2 days prior to ovulation, etc. (Modified with permission from Bahr *et al.*, 1983.)

and collected whole clutches on the day that the last egg was laid. All of the eggs were frozen whole and stored at  $-20^{\circ}$ . Only one egg per clutch was analyzed in this investigation, and it was selected randomly with respect to its position in the laying order. Incubation in both the dark-eyed junco and the red-winged blackbird typically does not begin until after the laying of the penultimate egg (personal observation); therefore, no egg, when collected, would have been incubated for longer than about 24 h. In the domestic chicken, embryos that have been incubated for 24 h have just completed the formation of the neural groove and are initiating differentiation of the mesoderm into somites and nephrotomes (Rol'nik, 1970). If we assume that the differentiation of dark-eyed junco and red-winged blackbird embryos proceeds at approximately the same rate in the early stages of development as that of the chicken, no steroidogenic tissues would yet have formed in the embryos at the time the eggs were collected; therefore, embryos could not have contributed to the hormone pool present in the yolk.

To prepare the yolks for hormone analysis, we first separated the yolk of each egg from the albumin by taking advantage of the fact that albumin thaws more quickly than yolk. The frozen yolks were then dissected with a razor blade, and samples of similar mass were taken from the interior, intermediate, and exterior layers of the yolk (Fig. 2). Sample masses were recorded immediately upon dissection and ranged from 5.1 to 17.9 mg; the largest difference in mass between samples from the same egg was 6.8 mg. Because hormone concentrations are expressed as picograms per milligram of yolk, variation in sample masses does not contribute to error in the measurement of hormone concentrations. After the masses of the yolk samples were recorded, individual samples were homogenized in 1.5-ml Eppendorf microcentrifuge tubes with 500  $\mu$ l of water using a Vortex mixer. Homogenization was facilitated by the addition of several glass beads to each tube.

### Radioimmunoassay

All samples from dark-eyed junco eggs were analyzed for the presence of progesterone,  $17\beta$ -estradiol, and testosterone with a competitive-binding radioimmunoassay, as outlined by Wingfield and Farner (1975). Samples from red-winged blackbird eggs were ana-

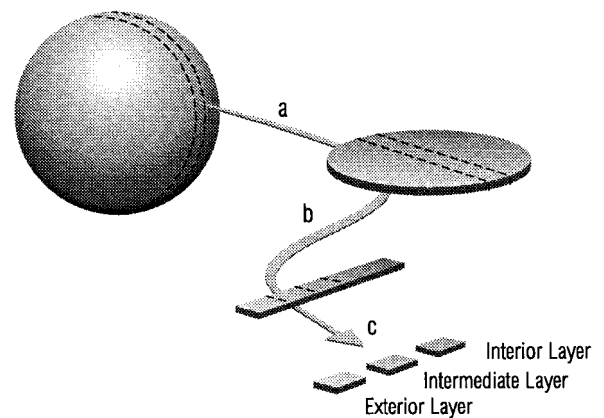


FIG. 2. A schematic representation of the dissection of interior, intermediate, and exterior layers from a whole frozen yolk. A disk approximately 1 mm in thickness passing through the core of the yolk was first removed (a). This disk was then reduced to a three-dimensional rectangular structure approximately 3 mm in width (b). Yolk samples of approximately equal mass were then excised from the interior, intermediate, and exterior portions of the yolk (c).

lyzed for the presence of testosterone, but not for progesterone or  $17\beta$ -estradiol. Two separate assays were performed, one for the dark-eyed junco samples and one for the red-winged blackbird samples. The details of this procedure are as follows: approximately 2000 cpm each of [ $^3$ H]progesterone, [ $^3$ H]testosterone, and  $17\beta$ -[ $^3$ H]estradiol (New England Nuclear Corp., Boston, MA) were added to each yolk sample to allow the calculation of recovery percentages following extraction and chromatography. Because only testosterone was measured in the eggs of the red-winged blackbird, yolk samples from that species received only [ $^3$ H]testosterone. The endogenous and tritiated steroids were extracted with petroleum and diethyl ethers (30%:70%), followed by a precipitation with 95% ethanol to remove excess lipids (Schwabl, 1993). The extracts were evaporated and redissolved in 10% ethyl acetate in isooctane before being applied to chromatography columns that consisted of a celite:ethylene glycol:propylene glycol upper phase and a celite:water lower phase. The progesterone fraction was eluted with 2% ethyl acetate in isooctane, the testosterone fraction with 20% ethyl acetate in isooctane, and the  $17\beta$ -estradiol fraction with 40% ethyl acetate in isooctane. Hormone concentration was measured by competitive-binding radioimmunoassay with tritiated hormone

and specific antibody. (Progesterone and testosterone antisera were purchased from Wien Laboratories, Succasunna, NJ, and  $17\beta$ -estradiol antiserum was from Arnel, New York, NY). Duplicate values of each sample were compared to a standard curve that ranged in concentration from 3.91 to 1000 pg for progesterone and from 500 to 1.95 pg for  $17\beta$ -estradiol and testosterone. Recovery values for the dark-eyed junco samples averaged 47% for progesterone, 71% for  $17\beta$ -estradiol, and 71% for testosterone. The average recovery value for testosterone in the red-winged blackbird samples was 65%. Intra-assay variation, which was calculated as the coefficient of variation of values obtained from standard samples of known concentration, was 19% for progesterone, 19% for  $17\beta$ -estradiol, 26% for testosterone in the dark-eyed junco samples, and 3% for testosterone in the red-winged blackbird samples.

### Statistics

We used a repeated measures analysis of variance to determine whether differences in progesterone and testosterone concentrations existed among layers within an egg. We used a Friedman repeated measures analysis of variance on ranks to determine whether  $17\beta$ -estradiol concentration varied among layers of yolk. A nonparametric statistic was used in the case of  $17\beta$ -estradiol because the sample population failed the Kolmogorov-Smirnov test for normal distribution. Multiple contrasts between layers were made for all three hormones using the Student-Newman-Keuls method.

We performed a Kruskal-Wallis analysis of variance on ranks to determine whether the average concentrations (calculated as the average of values from all of the layers) of progesterone,  $17\beta$ -estradiol, and testosterone in the dark-eyed junco eggs differed from one another. Again, nonparametric statistics were used because tests for normality failed. The Student-Newman-Keuls method was used to make multiple comparisons among the three treatment groups.

## RESULTS

Progesterone concentrations in the eggs of dark-eyed juncos averaged ( $\pm 1$  SE)  $147.6 \pm 29.1$ ,  $205.7 \pm$

$47.5$ , and  $982.1 \pm 122.3$  pg/mg of yolk for the interior, intermediate, and exterior layers, respectively, and increased significantly from the interior to the exterior of the yolk ( $F_{2,20} = 49.9$ ,  $P < 0.001$ , Fig. 3). Pairwise multiple comparisons revealed that progesterone con-

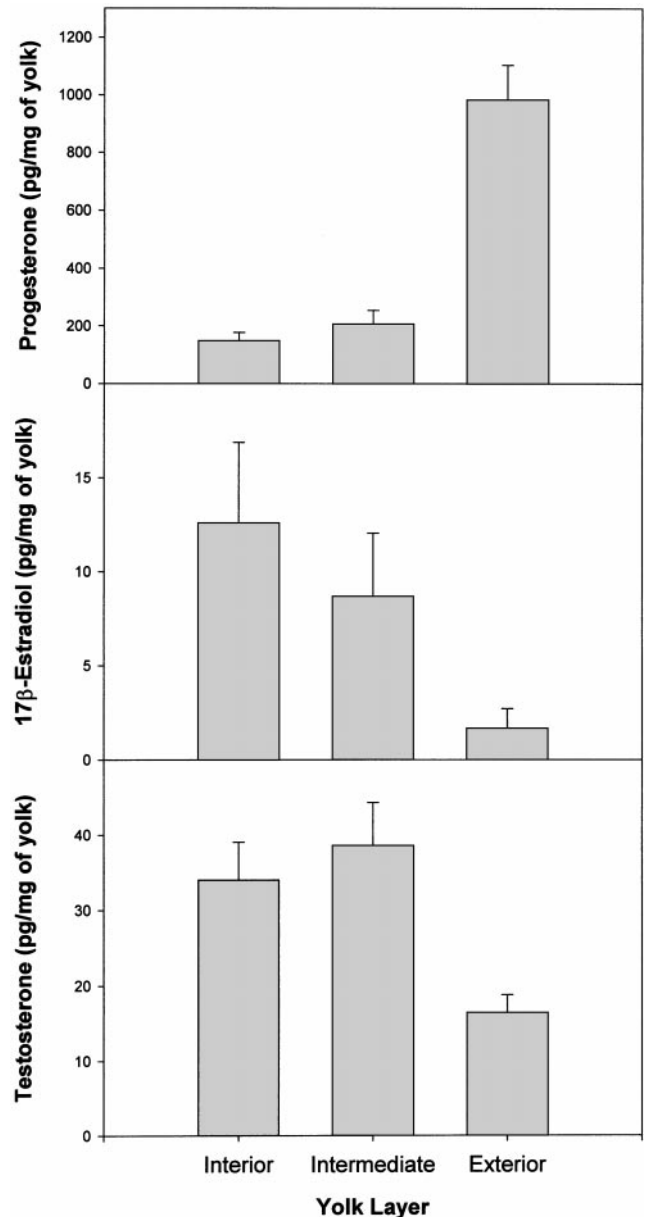


FIG. 3. Mean progesterone,  $17\beta$ -estradiol, and testosterone concentrations in interior, intermediate, and exterior layers of yolk in eggs of the dark-eyed junco. Error bars represent 1 SEM. A repeated measures ANOVA indicated significant differences among the layers ( $P < 0.001$ ,  $n = 7$ ) for all three hormones.

centration in the exterior layer was significantly higher than those in the interior and intermediate layers ( $P < 0.05$ ). In contrast,  $17\beta$ -estradiol concentrations averaged  $12.6 \pm 4.3$ ,  $8.7 \pm 3.4$ , and  $1.7 \pm 1.1$  pg/mg of yolk for the interior, intermediate, and exterior layers, respectively, and decreased significantly from the interior to the exterior layers of the yolk ( $\chi^2 = 14.0$ ,  $P < 0.001$ ,  $df = 2$ , Fig. 3). The concentration of  $17\beta$ -estradiol in each of the layers was significantly different from the concentration in each of the other layers ( $P < 0.05$ ). Testosterone concentrations averaged  $34.0 \pm 5.0$ ,  $38.6 \pm 5.7$  and  $16.4 \pm 2.4$  pg/mg of yolk for the interior, intermediate, and exterior layers, respectively, and remained constant from the interior to the intermediate layers of the yolk before dropping sharply between the intermediate and exterior layers ( $F_{2,20} = 36.2$ ,  $P < 0.001$ , Fig. 3). Testosterone concentration in the exterior layer was significantly lower than the concentrations in both the interior and intermediate layers ( $P < 0.05$ ), which did not differ from one another.

Testosterone concentrations in the eggs of red-winged blackbirds averaged  $34.4 \pm 4.6$ ,  $37.8 \pm 4.8$ , and  $21.6 \pm 3.4$  pg/mg of yolk for the interior, intermediate, and exterior layers, respectively, and also remained constant from the interior to the intermediate layers of the yolk before dropping sharply between the intermediate and exterior layers ( $F_{2,50} = 32.7$ ,  $P < 0.001$ , Fig. 4).

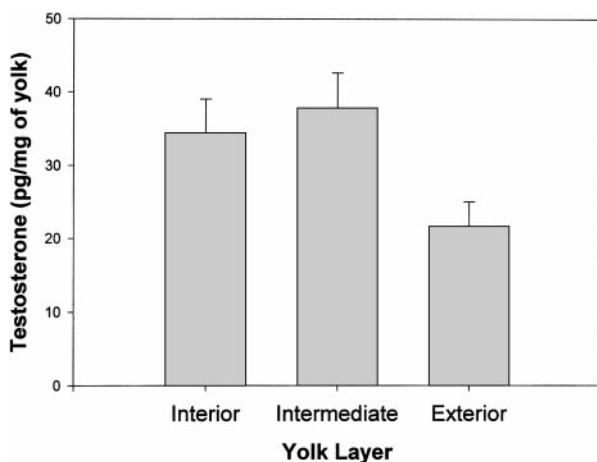


FIG. 4. Mean testosterone concentration in interior, intermediate, and exterior layers of yolk in eggs of the red-winged blackbird. Error bars represent 1 SEM. A repeated measures ANOVA indicated significant differences among the layers ( $P < 0.001$ ,  $n = 17$ ).

Pairwise multiple comparisons using the Student–Newman–Keuls method revealed that, as in the eggs of the dark-eyed junco, testosterone concentration in the exterior layer was significantly lower than in both the interior and the intermediate layers ( $P < 0.05$ ), which did not differ from one another.

In the dark-eyed junco, the absolute concentrations of progesterone,  $17\beta$ -estradiol, and testosterone (calculated as the average of values from all of the layers) differed significantly from one another ( $H = 50.4$ ,  $P < 0.001$ ,  $df = 2$ ). The means and standard errors of the concentrations of the three hormones were  $445.12 \pm 95.09$  pg/mg of yolk for progesterone,  $29.64 \pm 3.31$  pg/mg of yolk for testosterone, and  $7.63 \pm 2.03$  pg/mg of yolk for  $17\beta$ -estradiol. Pairwise multiple comparisons revealed significant differences ( $P < 0.05$ ) in all cases.

## DISCUSSION

As predicted, we found significant variation in the concentrations of progesterone,  $17\beta$ -estradiol, and testosterone among the yolk layers of individual eggs. Moreover, the patterns of hormone deposition matched those predicted by previous studies of the temporal patterns of steroidogenesis in the follicle of the chicken (Bahr *et al.*, 1983). These results indicate that steroid concentrations in the yolk vary in parallel with steroid concentrations in the follicle of the laying female. Regardless of the maternal source of hormone, the presence of this variation in the concentrations of steroids has implications for the reliability of yolk sampling techniques and for the timing of embryonic exposure to sex steroids.

### *The Relationship between Yolk Steroid Concentrations and Follicular and Plasma Steroid Concentrations in the Female*

In the domestic hen the follicular concentrations of progesterone,  $17\beta$ -estradiol, and testosterone change independently throughout the period of yolk formation (Bahr *et al.*, 1983; Fig. 1). The profiles of progesterone,  $17\beta$ -estradiol, and testosterone within the yolks of dark-eyed juncos and red-winged blackbirds correspond to the temporal changes in steroid production

that occur in the chicken. These results suggest that the production of steroids in the follicles of dark-eyed juncos and red-winged blackbirds during yolk formation may resemble that of the chicken.

It is likely that yolk steroids play a role in chick development and are not simply by-products of the physiological processes of the female, i.e., steroidogenesis and yolk deposition. Indeed, Schwabl demonstrated in the canary (*Serinus canaria*) that between-egg variation in yolk testosterone concentration is correlated with nestling growth rates (1996b) and with juvenile social rank (1993). If variation in hormone concentration among eggs influences the reproductive success of females, then we would expect that selection would favor mechanisms that regulate or adjust the amount of hormone that is delivered to individual eggs. Similarly, we would expect mechanisms that regulate the amount of hormone that is delivered to layers within any individual egg, particularly if variation in steroid concentration within an egg contributes to the fitness of offspring.

Studies of female domestic hens and of quail (*Coturnix coturnix japonica*) have indicated that the hormonal profile of the follicle does not necessarily mirror the hormonal profile of the circulating plasma (Doi *et al.*, 1980; Hammond *et al.*, 1980). If steroid concentrations in the follicle and the plasma can vary independently, then one might ask whether hormonal concentrations within any particular yolk are more directly influenced by hormonal concentrations in the follicle, which envelops the yolk during its growth phase, or by hormonal concentrations in the plasma, which flows through the follicle and supplies proteins and lipids from the liver to the maturing ovum. If we assume that steroidogenesis in the follicles of the dark-eyed junco and the red-winged blackbird is similar to that of the chicken, then our results suggest that the follicle is the primary influence on the pattern of steroid deposition within an individual yolk. The partial separation of follicular and circulating plasma steroid levels could be a mechanism that has evolved to help control the amount of hormone that is delivered to eggs.

Previous studies have found that the concentration of yolk testosterone increases with laying order in the canary (Schwabl, 1993), the red-winged blackbird (Lipari *et al.*, 1999), and the dark-eyed junco (unpublished data). One possible explanation for why testosterone is

elevated in later-laid eggs is that there is a progressive elevation in female plasma testosterone levels during the egg-laying stage (Wingfield and Farner, 1978; Donham, 1979; Hegner and Wingfield, 1986), perhaps reflecting the summation of follicular contributions. Because yolk is deposited in concentric spheres at the periphery of the yolk, one would also expect testosterone concentration to increase from the interior to the exterior layers of the yolk. However, this does not happen as evidenced by the results presented here. Therefore, an alternative explanation is required. Shababi *et al.* (1975) found in the domestic hen that there are intrinsic differences among follicles collected from the same bird in the amount of testosterone that they produce. Follicles that mature into earlier-laid eggs within a clutch produce significantly lower amounts of testosterone than those that develop later in the laying order. It may be that intraclutch variation in yolk testosterone concentration in the canary and the red-winged blackbird is a result of the increase in follicular production of testosterone that is correlated with the order of follicle maturation. This variation in hormone production at the level of the follicle could represent a second mechanism that has evolved to control the amount of hormone the female transfers to her eggs.

Intraclutch variation in testosterone concentration is also found in cattle egrets (*Bubulcus ibis*; Schwabl *et al.*, 1997), but it differs from that of the canary and the red-winged blackbird. The testosterone concentration of cattle egret eggs decreases with laying order, a pattern that is presumably linked to the fact that in this species the last-hatched young is often subjected to high levels of sibling aggression, sometimes with fatal results (Ploger and Mock, 1986; Schwabl *et al.*, 1997). We predict that in the cattle egret the follicles that produce later-laid eggs would produce less testosterone than those that produce first-laid eggs.

### **Implications for Yolk Sampling Techniques**

To assess the effects of yolk steroid hormones on development and on fitness, it is necessary to investigate the relationship between yolk steroid levels and certain behavioral and physiological parameters of hatchlings. One technique that has been used to accomplish this is to take biopsies of yolk from viable eggs and then to measure selected traits of the birds that are produced from those eggs. For example, Schwabl

(1993) collected yolk samples from viable canary eggs and found a positive correlation between testosterone concentration and juvenile social rank. The removal of yolk samples requires the insertion of a needle through the shell and albumin and into the yolk. We have found that there is variation in steroid concentration across the layers of a yolk; therefore, the accuracy of any hormonal information gathered from yolk samples acquired in this way would depend on the placement of the point of the needle within the yolk. In light of this finding, we would caution that care be taken in obtaining yolk samples so that samples are consistently collected from the same location within the yolk. Because eggs are usually placed over a fiberoptic light source when samples are taken, one should be able to successfully position the biopsy needle by sight alone.

### ***Implications for Developmental Effects of Steroid Hormones***

Steroid hormones in avian egg yolks have been shown to have developmental effects on the embryos that are produced. As already noted, the social rank of juvenile canaries is positively correlated with the concentration of yolk testosterone in the eggs from which they hatch (Schwabl, 1993). Schwabl (1996b) also found that the injection of exogenous testosterone into the yolks of canary eggs enhances nestling growth rates (measured as body mass and tarsus length), accelerates the development of eye slits, and increases the likelihood that nestlings will beg immediately after hatching. Our finding that hormone concentrations vary within the yolks of individual eggs suggests that developing embryos may be exposed to different steroid levels as development proceeds.

The utilization of yolk by developing embryos is accomplished by the formation of the yolk sac, a temporary embryonic organ that encapsulates the yolk and is connected to the embryo via a system of vitelline arteries and veins (Rol'nik, 1970). These blood vessels cover the sac and are responsible for the transport of yolk substances to the circulatory vasculature of the embryo. There are two separate mechanisms by which yolk is absorbed into the yolk sac (Rol'nik, 1970). The first of these is phagocytosis of yolk granules, a process that may be facilitated by the presence of folds on the internal surface of the yolk sac. The second mechanism involves the secretion of proteolytic and lipolytic

enzymes by the yolk sac into the yolk. These enzymes break down yolk globules for transfer to the embryonic vasculature through the epithelium of the yolk sac.

Both endocytosis and enzymatic catabolism of yolk globules occur at the interface of the yolk and the yolk sac, which might suggest that absorption of the yolk proceeds from the exterior to the interior of the yolk. If true, then variation in the concentration of steroid hormones among yolk layers, such as we report here, would lead to corresponding temporal variation in the availability of those hormones during embryonic development. However, the absorption of the yolk probably does not proceed in so simple a manner. First, in the chick embryo the maximum activity of proteases occurs on the 10th day of development, while lipase activity reaches a maximum on day 16 (Rol'nik, 1970). These maxima coincide with peaks of protein and lipid metabolism by the embryo, therefore suggesting that different components of the yolk may be selected for absorption independently of one another. Second, in the ostrich (*Struthio camelus*) there is extensive mixing of the yolk during the middle and late phases of incubation (J.M. Starck, personal communication). If these processes occur in all avian species, then the interlayer variation in steroid concentrations that is found in freshly laid eggs may not persist throughout the embryonic period. Whether the continuity of layer variation in steroid hormones persists throughout embryonic development must be clarified before inferences about the developmental effects of yolk steroid hormones can be made. Our study could be extended by selecting eggs at various times during development and analyzing them for variation in steroid content.

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